Arizona Iceberg Lettuce Research Council
Project Annual Report: July 2004 – September 2005

Project Title: Detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed and soil

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Research Locations: Department of Plant Pathology, University of Arizona, Tucson and various fields in the winter lettuce producing area around Yuma, AZ.

Purpose of the Study

In 2001, the disease Fusarium wilt of lettuce, caused by the fungus *Fusarium oxysporum* f.sp. *lactucae*, appeared in 5 fields near Wellton, Arizona. In 2002, the disease was reported in additional fields in the Gila Valley and near Yuma, and it became apparent that the pathogen was spreading in the prime winter lettuce growing region of Arizona. Because this is a relative new disease, very little is known about the etiology and epidemiology of Fusarium wilt, and few options are currently available to growers to manage it. Therefore, comprehensive research program was initiated in order to develop basic understanding of the biology and genetics of the fungus. In this study, several objectives were developed. The phylogeny of this fungus relative to other strains of *Fusarium oxysporum* was determined using five different genomic regions. Only one of these could resolve the lactucae isolates into a clade distinct from other *Fusarium oxysporum* isolates. Based on this phylogeny, we are developing a DNA-based method to detect the fungus in both seed and soil. In addition, studies have been initiated to monitor the movement of the pathogen in the lettuce growing areas around Yuma based upon DNA fingerprint analyses.

Project Objectives:

- Develop DNA-based pathogenic PCR primers to detect the pathogen and not other related species
- Develop a seed assay to detect the pathogen in seed
- Screen commercial seed for the presence of the pathogen
- Perform greenhouse-based studies to investigate the production of infested seed from infected lettuce plants
- Develop a soil assay to detect the pathogen in field soil (and also in lettuce tissue)
- Examine population genetic diversity of the pathogen to understand movement within Arizona and between California and Arizona

Results:

Objective 1.

We have found a region of DNA in the *Fusarium oxysporum* f.sp. *lactucae* genome with sequences unique to the fungus causing *Fusarium* wilt in lettuce: the nuclear intergenis spacer region (IGS). Based upon DNA sequences from this region, we have developed PCR primers that detect only lettuce-pathogenic isolates of *Fusarium* DNA in the presence of much larger amounts of other non-pathogenic *Fusarium* DNA. However, the initial primers produced considerable background signal and could amplify serially diluted genomic DNA at an amount of only 20 picograms. The sensitivity of the method was improved with the design of an additional primer with a 3' one-base difference. Using this primer, genomic DNA amounts as low as 2 femtograms could be detected (Figure 1). In specificity tests, DNA was amplified from the *lactucae* isolates and not from other *Fusarium* isolates and species (Figure 2).

Objective 2:

Based upon the unique DNA sequences, a three-step PCR-based detection method for *F. o*. f. sp. *lactucae* has been developed which utilizes an initial PCR amplification, followed by a restriction digest of the amplified fragment, followed by a second PCR amplification. Using this method, we were able to detect pathogenic *Fusarium* DNA in extracts of lettuce seed that have been artificially inoculated with target DNA. In these experiments, the DNA was amplified from seeds with 0.1% artificial infestation (Figure 3).

Objective 3:

25 commercial lots were obtained from commercial seed companies in 2003-2004. 18 of these lots were examined using our DNA-based seed assay method. Each assay examined 1 gram of seed (approx. 800-1000 seeds) at a time. For each lot, 5 grams of seed were examined (5 replications = approx. 5000 seeds). Initially, 5 of the commercial lots tested were positive for the presence of *lactucae* using our DNA-based method (PCR). However, using a direct seed plating method utilizing a selective agar medium all lots were negative for the pathogen, suggesting that our original analysis resulted in false positives. In 2004-2005, 20 commercial lots were obtained from commercial seed companies and these lots also tested negative for the pathogen using both the PCR method and the direct seed plating method. These are preliminary results and all tests are currently being repeated to confirm findings. Any suspect *Fusarium* isolates recovered from these lots will be tested for pathogenicity on susceptible lettuce cultivars to determine if they are in fact *F. oxysporum* f.sp. *lactucae*.

Objective 4:

Studies were performed in the greenhouse to determine if seed could naturally become infected. Both head and leaf cultivars were used and plants were inoculated at the roots. The movement of the pathogen was monitored up the flower stalk and into the seed. When the pathogen was introduced to lettuce roots during transplanting, it could usually be recovered all the way up the flower stalk to the flower pedicel at lettuce maturity, but usually not from the seed (Table 1). However, in 2% of the inoculations the pathogen was recovered from the seed as well, confirming that infected seed can be produced from infected plants in some cases. In addition, when uninfected seed was mixed with infected plant material and then cleaned, the resulting seed was usually infested, confirming that seed can easily become infested during the threshing

process. Important to note is that most cultivars tested were moderately tolerant to the fungus and did not show significant disease symptoms. However, the fungus grew and multiplied well in these plants and moved throughout the tissue. Thus, even though tolerant cultivars may be planted for seed production, they can still produce infested seed which can result in the further spread of the pathogen in lettuce fields when the seed is used.

Objective 5:
Soil samples of two infested field from Wellton were assayed using the designed primers. After genomic DNA isolation from the soil samples, serial dilutions were made with pure DNA from one of the *lactucae* samples. The *lactucae* DNA was amplified from three of the six samples that contained respectively 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} ng/µl of pure DNA but not from the unspiked sample implying that the primers can detect the target DNA in soil containing the fungus (Figure 6).

Ojective 6:
Population studies were conducted to characterize variation among *lactucae* isolates and changes in populations in AZ over time. These studies were based upon DNA fingerprint analysis, rather than DNA sequence analysis used in the systematic study. Fingerprint analysis can differentiate individual genotypes of *lactucae* and determine the distribution of these across specific locations. Most of the AZ isolates (n = 40) were identical and these were recovered in 2002 and matched exactly the HL1 and HL2 isolates from the original CA infestation in 1990 (Figure 7). Interestingly, these also matched the Italy isolates recovered in 2002. A few AZ isolates recovered in 2003 and 2004 were new genotypes and could represent new introductions or forms of the fungus (Table 2). So far, the isolates used in the population studies represent four genotypes (Table 3). Total CA isolates (n = 11) are much more diverse than total AZ isolates (n = 40).

Problems encountered: Additional specificity screens including several other fungi revealed that the original PCR primers cross-reacted with common saprobic *Alternaria alternata* species as well as certain isolates of *F. culmorum* and *F. concolor*. Upon further examination, we found that these fungi have a conserved *Hind III* restriction site and that our original detection method resulted in the amplification of the same size DNA fragments from all of them. Thus, there was the possibility of generating false positives if the samples to be screened contained spores of these fungi, and we conclude that the results obtained using our first primer sets for the commercial seed assays were indeed false positives. Realignment of the 5' and 3' ends of the IGS region was repeated which included sequences from these other fungi and different primer sets have been redesigned. Of these, the primer set FOL1/R4 was selected for the 1st PCR primers as it provided the most specific and sensitive detection of *Fusarium* (Figure 4). In serial dilution of genomic DNA from *lactucae* isolates, the lowest level of DNA detected using this primer set was 100 femtograms (Figure 5). Plans to improve on the sensitivity will include comparing several annealing temperatures, (60 °C, 62 °C, 65 °C, and 68° C) and annealing times.

Future Perspectives
Using the new primer set, we are currently re-assaying the commercial seed lots for the presence of the pathogen and we intend to use only primer pair FOL1/R4 as the 2nd PCR primers in the

assays. However, the FOL1/R4 primers still cross-reacts with two of the non-*lactucae* *Fursarium* species. Thus, there may be rare instances in which we still obtain a false positive result. We are still trying to remove this cross-reactivity, however, as it currently stands the method still is a very useful method as a negative screen. For seed lots that do result in a positive reaction, we shall sequence the fragment to conclusively determine if it is indeed *lactucae* DNA.

The method developed for assaying seeds for the presence of the pathogen also works well for soil assays. We are positive that the single new primer will work for soil assays too since it will be used in combination with the original FOL1 primer. The present challenge is to improve on its sensitivity such that it can detect the fungus in soil at very low inoculum concentrations. The limit of detection will be determined using soil from heavily infested fields after soil plate methods would have conclusively determined the presence of the pathogen. All fields from which the pathogen has been documented to-date will be subsequently sampled and soil dilutions will be tested to generate population data based upon densities. Following the determination of natural range of population densities, subsequent studies will examine the spatial distribution of the pathogen across fields.

Research will continue to evaluate population variation among isolates of *Fusarium* from newly infested fields in order to genotype any new introductions or changing strains. These studies are ongoing as new isolates are provided to us by Dr. Mike Matheron.

Appendix

Figure 1: Amplification of serially diluted DNA from *F. oxysporum* f.sp. *lactucae*. *A.* improved sensitivity with new primer. B. Level of sensitivity with originally designed primer set (note the background noise and limit of detection).

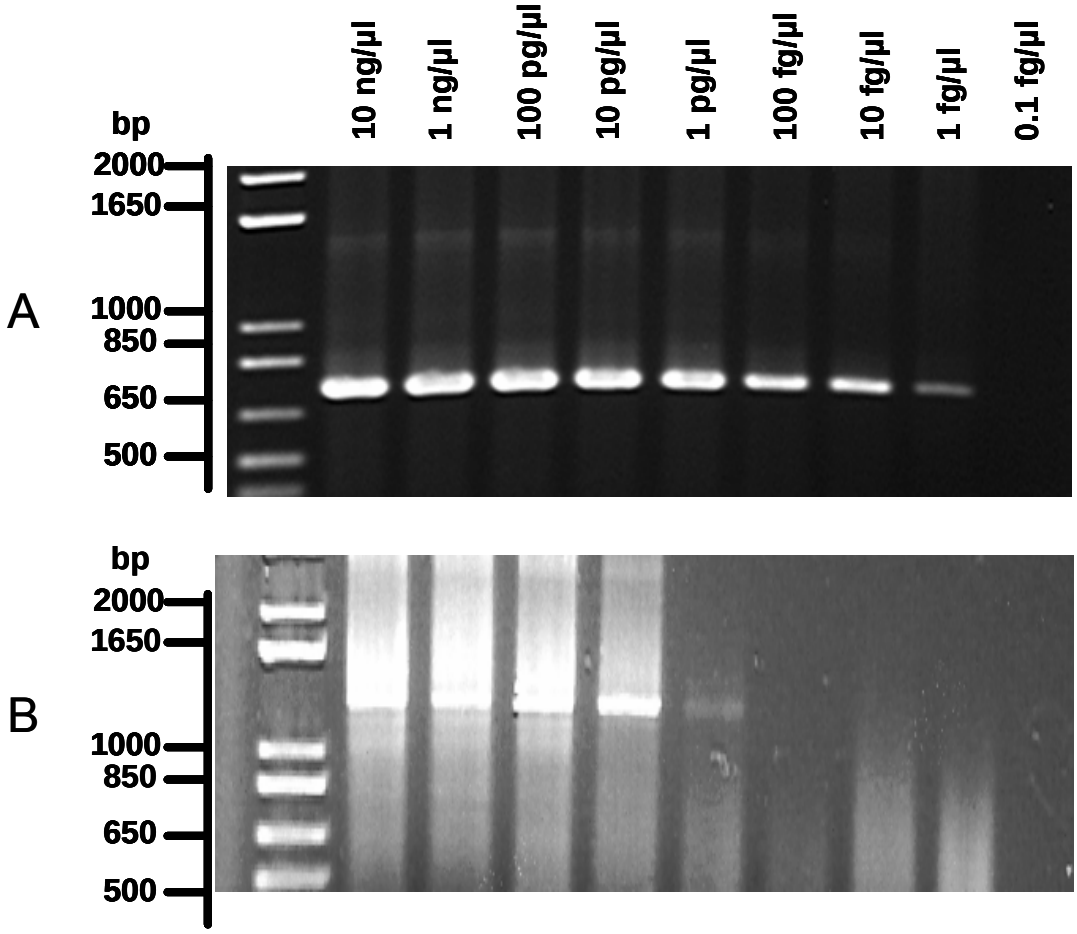


Figure 2. Specificity of primers: amplification of DNA from *lactucae* isolates but not from other *Fusarium* isolates and species.

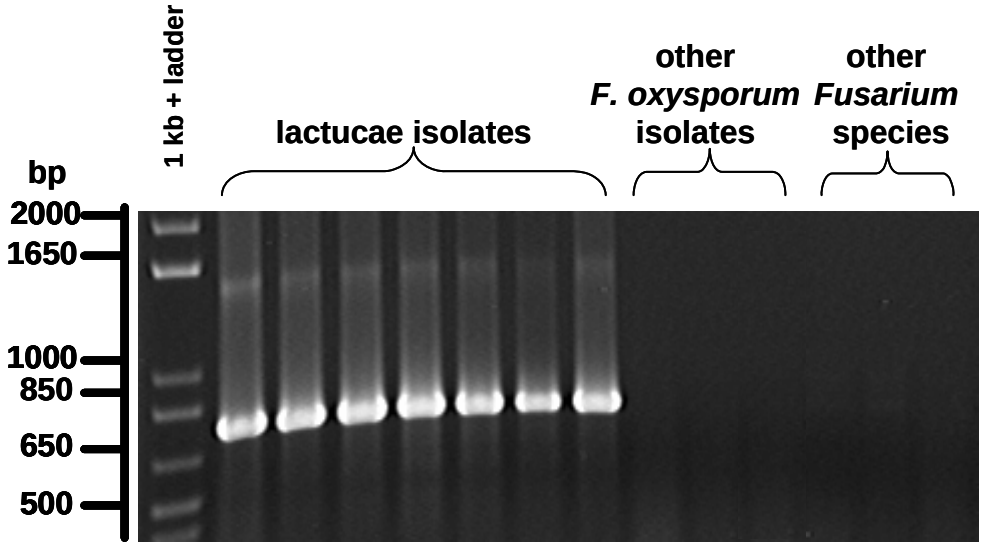


Figure 3 Limit of detection in artificially infested seeds. Amplification from seeds with different levels of artificial infestations. Target could be detected in seed with infestation level as low as 0.1%.

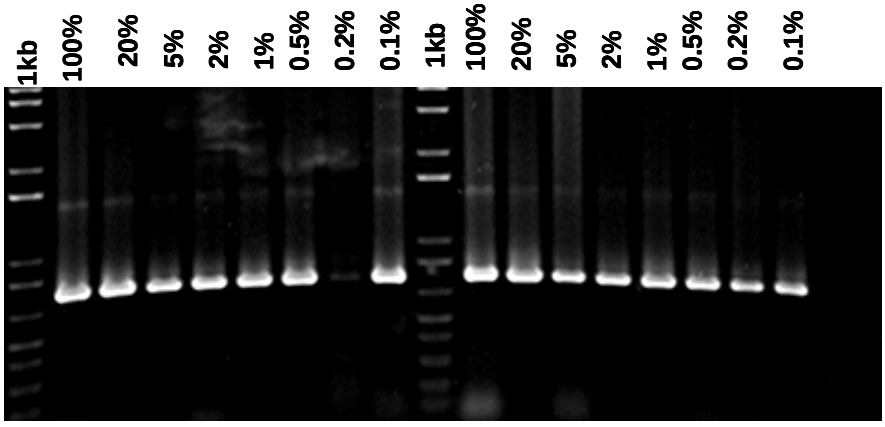
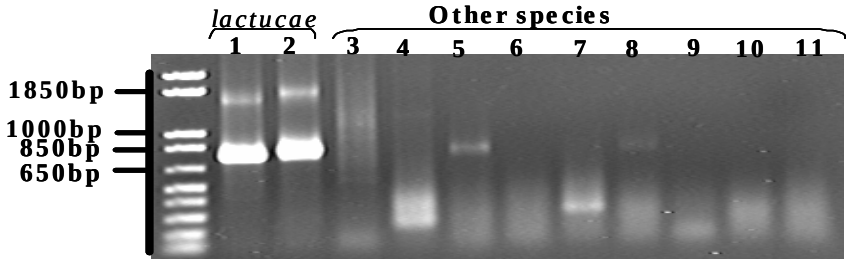
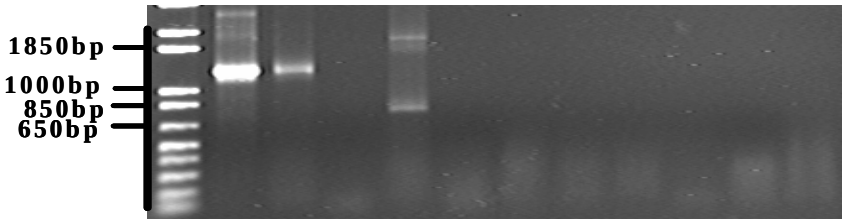


Figure 4: Specificity of new primers: A. primer pair FOL1/HO amplified the DNA of the target size from *lactucae* isolates (Lane 1 and 2) and also from *F. culmorum* (lane 5), *F. concolor* (lane 8) but not other *Fusarium* isolates and species. B. Primer pair HO/R4 amplified the target fragment only from *lactucae forma specialis*. In both figures, the order of the samples is the same and lanes 9 and 11 correspond to samples from the *Alternaria alternata* species group. Our assay does not currently amplify DNA from this fungus as it did in earlier experiments.

A.





B.

Figure 5: Amplification of serially diluted DNA from *F. oxysporum* f. sp. *lactucae*. **A**. Better sensitivity with primer FOL1 (the limit of detection was 100fg/µl). **B**. Less sensitivity with primer R4 (limit of detection is 10pg for this primer pair and the band is barely visible on this gel).

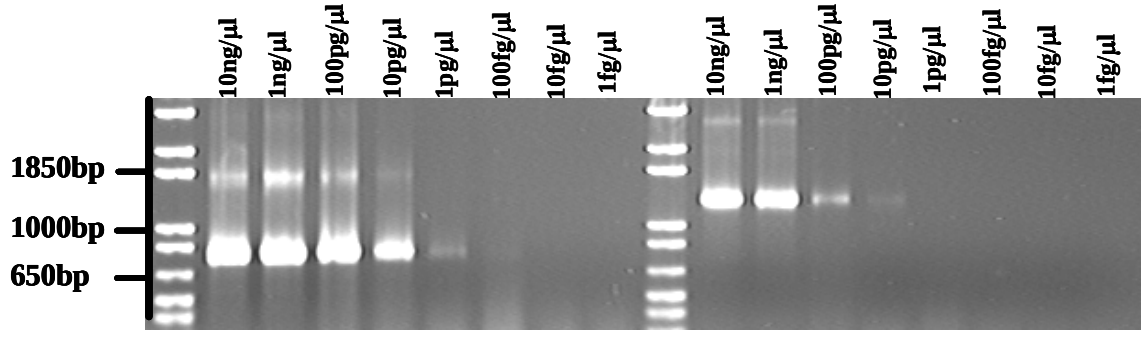


Figure 6. Soil assay using primers specific for *Fusarium oxysporum* pathogenic on lettuce. Soil sample obtained from an infested field in Wellton. Six independent DNA isolations were done on 1g each of soil sample. The samples were bulked and spiked with 2 µl of serially diluted genomic DNA from a pathogenic isolate. The lanes are as follows: Lane 1 is unspiked with purified DNA; 2-7 were spiked with decreasing amounts of purified genomic DNA and 8 represents genomic DNA from a pathogenic isolate.

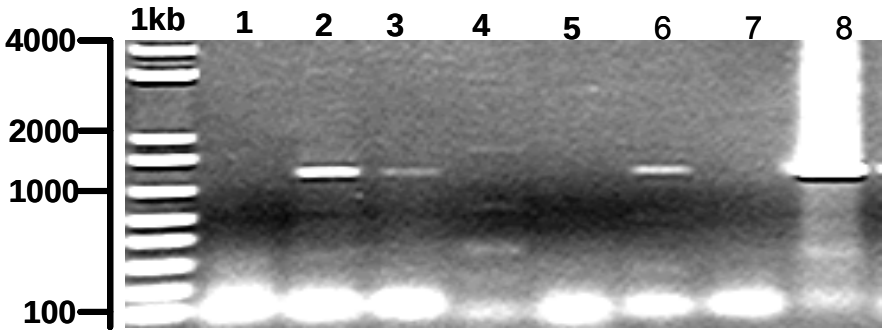


Table 1: Recovery of *Fusarium oxysporum* f. sp. *lactucae* from lettuce plants at maturity following inoculation during transplanting. Recovery was based upon a standard agar plating technique using Komada's medium. The pathogen was not detected on seed using this technique, but was detected using the PCR method in two of the samples.

Cultivar	Type of inoculation	Plant Parts				
		Basal Nodes	Middle nodes	Upper node	Flower pedicel	Seed (PCR method)
Sharpshooter	Root dip	+	+	+	+	-
	Soil inoc.	+	+	+	+	+
King Louie	Root dip	+	+	+	+	-
	Soil inoc.	+	+	+	+	-
Vulcan	Root dip	+	+	+	+	-
	Soil inoc.	+	+	+	+	+

Table 2. Genotyping of representative *Fusarium oxysporium* f. sp. *lactucae* isolates using microsatellite-primed PCR fingerprint patterns.

	Microsatellite Primers			
***Lactucae* Isolate**	**$[GTG]_5$**	**T3B**	**M13**	**$[GACA]_4$**	**ACA_5**
HL2	**A**	**A**	**A**	**A**	**A**
2002LR2	**A**	**A**	**A**	**A**	**A**
2002JCP007	**A**	**A**	**A**	**A**	**A**
2002-01	**A**	**A**	**A**	**A**	**A**
2002Y05	**A**	**A**	**A**	**A**	**A**
2003Y01	**A**	**A**	**A**	**A**	**A**
2003-08	**A**	**A**	**A**	**A**	**A**
2003-07	**A**	**A**	**A**	**A**	**A**
2004-04	**B**	**B**	**B**	**B**	**B**
2004-03	**C**	**A**	**C**	**C**	**A**
2004-05	**A**	**A**	**A**	**A**	**A**
2003-09	**A**	**C**	**D**	**A**	**A**
2004-02	**A**	**C**	**D**	**A**	**A**
2003Y06	**A**	**A**	**A**	**A**	**A**
2003Y03	**A**	**A**	**A**	**A**	**A**
2003-02	**A**	**A**	**A**	**A**	**A**

Table 3. Genotypes obtained from the fingerprint patterns of the isolates

Genotype	**# of Fields**	**Primers**				
		1	**2**	**3**	**4**	**5**
1	**24**	**A**	**A**	**A**	**A**	**A**
2	**01**	**B**	**B**	**B**	**B**	**B**
3	**01**	**C**	**A**	**C**	**C**	**A**
4	**02**	**A**	**C**	**D**	**A**	**A**

Figure 6: Distribution of genotypes in lettuce fields in AZ 2002-2004. Genotypes designated by colored ovals: white, red, green. Position of oval represents location of field where genotype was recovered. Note: Stars represent location of infested fields still requiring pathogen isolation and genotyping.